STUDIES OF MEMBRANE FUSION.

V. FUSION OF ERYTHROCYTES WITH NON-HAEMOLYTIC SENDAI VIRUS

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SUMMARY

The fusion of human erythrocytes with non-haemolytic '1-day' Sendai virus has been studied by electron microscopy. The mechanism of viral envelope-cell fusion is the same as that described previously for haemolytic '3-day' Sendai virus except that fusion is frequently arrested at an initial stage when 2 segments of smooth linear viral membrane fuse and become incorporated into the erythrocyte membrane. After longer periods of incubation at 37 °C, in addition to many partly fused virus particles, long (up to 4 μm) lengths of smooth linear viral membrane are seen within the erythrocyte membrane which arise by linear aggregation of shorter (~ 0.25 μm long) segments of smooth linear membrane derived from individual fused viral envelopes. Cell–Cell fusion, as a result of the fusion of a viral envelope with 2 adjacent erythrocytes also occurs but, in the absence of cell swelling, fusion is arrested at this stage with cells joined by one (or more) small cytoplasmic bridges. Typical fused cells are produced if such cells are swollen with hypotonic buffer. These observations provide further evidence that membrane fusion and cell swelling are distinct events in cell fusion and that cell swelling is the driving force both for completing the incorporation of the viral envelope into the cell membrane and for expanding cells connected by small cytoplasmic bridges to form spherical fused cells. Little lateral diffusion of viral envelope components occurs in the absence of cell swelling; in fact, some aggregation of components occurs. Comparison with previous studies using haemolytic '3-day' Sendai virus suggests that virally induced cell swelling perturbs membrane structure so as to allow the rapid lateral diffusion of integrated viral envelope components.

INTRODUCTION

Previous morphological and biochemical studies have led to the conclusion that Sendai virus-induced cell fusion involves 3 distinct stages. Virus particles (1) bring adjacent cells into close contact (i.e. cause cell agglutination), (2) induce membrane fusion and the formation of small cytoplasmic bridges between cells, and (3) expand such cytoplasmic bridges to form spherical fused cells by a process of cell swelling (Knutton, 1978). Stage 1 occurs as a result of the interaction between the viral envelope haemagglutinin spike and sialic acid-containing receptors on the cell surface (Bächi, Deas & Howe, 1977). Stage 2 involves fusion of viral envelopes with the cell plasma membrane and cytoplasmic bridge formation occurs when there is simultaneous fusion between a virus and two crosslinked cells (i.e. formation of a cell–viral envelope-cell bridge) (Knutton, 1977, 1978). Stage 3, which involves virally induced cell swelling, results from the fact that many virus particles in virus preparations harvested following a 72-h infection of embryonated eggs ('3-day virus'), the virus preparation routinely used to fuse cells, have 'damaged' permeable envelopes. Fusion
with a cell makes the cell membrane permeable to low molecular weight compounds and ions (Pasternak & Micklem, 1973). Loss of cation asymmetry leads to an influx of water, with resultant swelling (Knutton et al. 1976) and, in the case of erythrocytes, cell rupture and haemolysis.

Recently it was shown that Sendai virus harvested following a single cycle infection of embryonated eggs ('1-day virus') was non-haemolytic (Homma, Shimizu, Shimizu & Ishida, 1976). Virus particles present in '1-day' preparations have intact envelopes (Shimizu, Shimizu, Ishida & Homma, 1976), do not induce a membrane permeability change on fusing with a cell membrane or cell swelling (Knutton 1978). Preliminary results using 1-day Sendai virus have shown that cytoplasmic bridge formation does occur but, in the absence of cell swelling, the fusion process is arrested at this stage (i.e. stage 2) (Knutton, 1978).

The mechanism of viral envelope–cell fusion, cell–cell fusion and the fate of viral envelope components under conditions where cell swelling occurs have already been established (Knutton, 1976, 1977, 1978, 1979). The use of ‘1-day virus’ allows one to study virus–cell fusion, cell–cell fusion and follow the fate of viral envelope components under conditions where cell swelling does not occur. In this paper I present morphological data which show that, although the mechanism of virus–cell fusion is identical to that previously described for '3-day virus' (Knutton, 1977), the subsequent fate of incorporated viral envelope components differs. With '1-day virus' there is little or no lateral diffusion of fused viral envelope components following viral envelope-cell fusion whereas the opposite is the case for '3-day virus' (Knutton, 1977, 1978; Bachi et al. 1977). These observations suggest that cell swelling perturbs, in some as yet identified way, the structural organization of the plasma membrane.

METHODS

Cell fusion

Human erythrocytes (Type O) from samples of blood freshly drawn into heparin were washed 3 times in Hanks' balanced salt solution (Hanks' BSS) (Hanks & Wallace, 1949). A 2 % suspension of washed erythrocytes was used. Non-haemolytic Sendai virus (Homma et al. 1976) was grown in 11-day-old chick embryos, harvested after 24 h and clarified by centrifugation. For fusion experiments 1 ml of virus (~ 10⁵ haemagglutinating units/ml (HAU/ml)) was added to 1 ml of the washed erythrocyte suspension at 4 °C. After 30 min the agglutinated cells were transferred to a waterbath at 37 °C and samples taken after various time intervals up to 2 h. Fusion was stopped either by the addition of an equal volume of 6 % glutaraldehyde in Hank's BSS or by chilling to 4 °C.

Electron microscopy

For scanning electron microscopy cells were allowed to settle on to gelatin-coated glass cover-slips (Vial & Porter, 1975) and immediately fixed with 3 % glutaraldehyde in Hanks' BSS for 30 min, postfixed for 1 h with 1 % buffered osmium tetroxide and dehydrated through a graded series of acetone solutions. The coverslips were then transferred to liquid carbon dioxide and critical point dried. Finally the coverslips were mounted on stubs, coated with a thin layer of gold, and the specimens examined in a Cambridge Type II stereoscan.

Freeze-fracture and thin-section microscopy were carried out as described in the preceding paper (Knutton, 1979).
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RESULTS

At 4 °C virus particles bind to the erythrocyte membrane and crosslinking of adjacent cells by virus particles causes cell agglutination (Fig. 1A). The uniformly small (~200 nm diameter) virus particles present in '1-day' preparations form deep invaginations in the erythrocyte membrane and are quite difficult to see by scanning microscopy even at high magnifications (Fig. 1B). Although somewhat distorted, agglutinated cells at 4 °C retain an essentially biconcave disk morphology.
Warming an agglutinated cell suspension to 37 °C triggers, after about 2–3 min, a change in viral envelope structure and fusion of viral envelopes with the erythrocyte membrane (Fig. 2). The changes in viral envelope structure and initial virus–cell fusion event are identical to those previously described for '3-day virus' (Knutton, 1976, 1977) and will not be described in detail here. In contrast to the fusion of '3-day' virions, many '1-day' virions do not become completely incorporated into the erythrocyte membrane and fusion is arrested after the initial stage when 2 smooth linear invaginations of the viral envelope (characterized in freeze-fracture replicas by smooth linear ridges in E fracture faces and linear grooves on P faces) have fused and become incorporated into the erythrocyte membrane (Fig. 2).

![Fig. 2. Freeze-fracture replica showing part of the fracture face (E) of an erythrocyte incubated with '1-day' virus at 37 °C for 5 min. Several virus particles, identified by pairs of parallel smooth linear ridges (arrows) can be seen to have fused with the erythrocyte membrane. × 48000.](image)

After longer (20–30 min) periods of incubation at 37 °C one sees, in addition to numerous fused and partially incorporated virus particles, long (up to 4 μm) lengths of smooth linear membrane (Fig. 3). The discontinuous structure of some of these long lengths of smooth membrane suggests that they are derived from shorter (∼0.25 μm) lengths of smooth membrane which are typical of those present in individual virus particles. Some images (Fig. 4) show that long lengths of smooth membrane are produced by a linear aggregation of shorter lengths derived from single fused virus particles. In this case aggregation of smooth E face ridges from 3 fused virus particles has occurred. In many instances the long ridges (Fig. 3A) and grooves (Fig. 3B) originate...
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from one of the pair of smooth linear invaginations of fused virus particles (Fig. 3, arrows).

The smooth linear viral ridges and grooves seen in freeze-fracture replicas represent linear invaginations of the viral envelope. When they are incorporated into the erythrocyte membrane and aggregate they produce similar linear invaginations of the erythrocyte membrane. This gives erythrocytes a highly convoluted profile when seen in both cross-fractured (Fig. 6) and cross-sectioned views (Fig. 5) and by scanning electron microscopy (Fig. 1 C, D). At this stage erythrocytes have become essentially spherical and, in addition to the long linear invaginations, many fused but incompletely incorporated virus particles are seen clearly in scanning micrographs (Fig. 1 C, D). Thin-section images also reveal nucleocapsids inside erythrocytes (Fig. 5, arrows).

Cross-fractures through erythrocytes which have been incubated at 37 °C show that, in addition to virus-cell fusion, cell-cell fusion has also occurred and many cells connected by small (~ 0.5 μm diameter) cytoplasmic bridges can be seen (Fig. 6, arrows). The curvature of the erythrocyte membrane at the sites of cytoplasmic bridges between cells is typical of the curvature of the viral envelope invaginations and suggests the involvement of fused virus particles in cytoplasmic bridge formation. Although such cells would not be recognized as fused by light microscopy, strictly speaking, such cells are fused since there is a cytoplasmic continuity between them. However, the term 'fused cell' is generally used to describe cells which have fused and become spherical as a result of swelling since such swollen fused cells can be readily recognized by light microscopy. Although cell swelling does not occur during fusion with '1-day virus' it is possible to detect which cells are fused (i.e. connected by cytoplasmic bridges) simply by swelling cells in hypotonic buffer. Then typical fused erythrocytes are seen (Knutton & Bächi, manuscript in preparation).

Fig. 3. Freeze-fracture replicas showing A, parts of the E and B, P fracture faces of erythrocytes incubated with '1-day' virus at 37 °C for 30 min. In addition to many fused virus particles (arrows) one sees long lengths of smooth linear ridge (r) on E faces (A) and complementary grooves (g) on P faces (B) which often originate from one of the pair of smooth ridges or grooves of individual fused virus particles (arrows). Cross-fractured views of fused virus particles indicate that many virions generate just 2 of the smooth linear infoldings required for fusion (arrowheads in A). A, × 26,300; inset, × 62,000; B, × 28,000.

Fig. 5. Thin-section images showing erythrocytes incubated with '1-day' virus at 37 °C for 30 min. Fused but incompletely incorporated virus particles (sv) can be seen. The numerous linear invaginations (arrows) give erythrocytes a highly convoluted profile. Viral nucleocapsids (nc) can be seen within erythrocytes usually in close association with the linear invaginations (inset), × 34,000; inset, × 50,000.

Overleaf.
Figs. 3A and 5. For legends see page 89.
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Figs. 3B and 4. For legends see pages 89 and 92.
Fig. 6. Freeze-fracture replica showing a cross-fracture through part of 3 erythrocytes which have been incubated with '1-day' virus at 37 °C for 30 min. Small (∼0.5 µm) cytoplasmic bridges (arrows) can be seen connecting the 3 erythrocytes. Viral envelope involvement in the formation of cytoplasmic bridges is suggested by the sharp curvature of the membrane (arrowheads) which is typical of cross-fractures through the linear viral invaginations. × 17,500.

Fig. 4. Freeze-fracture replica showing part of the E face of an erythrocyte incubated with '1-day' Sendai virus at 37 °C for 10 min. The long lengths of smooth linear ridge (arrow) appear to arise by linear aggregation of shorter lengths of ridge derived from individual fused virus particles. Several unfused virus particles (∇) can also be seen. × 35,000.
DISCUSSION

The observations reported here support previous conclusions that membrane fusion and cell swelling are distinct events in cell–cell fusion (Knutton, 1978). Fusion of '1-day' virions with the erythrocyte membrane does not induce a membrane permeability change or cell swelling and so cell–cell fusion is arrested at a stage when cells are connected by small cytoplasmic bridges. The presence of smooth linear viral membrane at such cytoplasmic bridges suggests that, as has already been shown to be the case for '3-day' virus (Knutton, 1977, 1978), cell fusion is mediated by a fused viral envelope. That typical fused cells can be produced from cells joined by small cytoplasmic bridges by swelling in hypotonic buffer (Knutton & Bachi, manuscript in preparation) is direct evidence that swelling is the driving force which expands such cells to form polykaryons. Complete incorporation of fused viral envelopes into the cell membrane also appears to require cell swelling. The presence of nucleocapsids inside intact erythrocytes, however, shows that cell swelling is not essential for virus infection.

The presence of smooth viral membrane incorporated into the erythrocyte membrane allows one to estimate the numbers of fused virus particles since most virus particles appear to generate just two of the ~0.25 μm long smooth membrane elements (Fig. 3A and inset, arrowheads). Fig. 3A, for example, represents approximately one tenth of the surface area of an erythrocyte and the total length of smooth viral membrane represents approximately fifty fused virus particles giving a figure of 500 virus particles fused with this particular erythrocyte.

Table 1. Biological properties of Sendai virus

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<th>'1-day' virus</th>
<th>'3-day' virus</th>
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<td>(a) Viral envelope-cell fusion</td>
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<td>(b) Cell–cell bridge formation</td>
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<td>(c) 'Permeable' envelopes</td>
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<td>(d) Induce a change in cell membrane permeability following fusion with the cell plasma membrane</td>
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<td>(e) Induce cell swelling</td>
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<td>(f) Induce erythrocyte haemolysis</td>
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<td>(g) Induce polykaryon formation</td>
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<td>(h) Rapid lateral diffusion of viral envelope components following virus–cell fusion</td>
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Table 1 lists some of the biological properties of '1-day' and '3-day' Sendai virus. The similarities (Table 1a, b) are related to viral envelope–cell fusion, the mechanism of which is identical. The differences (Table 1, c–h) all appear to be related to the fact that '3-day' virus preparations induce cell swelling whereas '1-day' virus preparations do not. Why virus particles harvested after 24 h have intact envelopes whereas after 72 h many virus particles have 'permeable' envelopes (Shimizu et al. 1976) is not understood. Physical damage to viral envelopes by freezing and thawing also makes
them 'permeable' (Shimizu et al. 1976). Consequently, frozen and thawed '1-day' virus behaves in an identical manner to '3-day' virus.

A comparison of the morphological characteristics of cells fused with '1-day' and '3-day' virus shows that, although the mechanism of viral envelope-cell fusion is the same, the fate of viral envelope components following fusion is very different. It is well documented that, following fusion of '3-day' virus with both erythrocytes (Bächli, Aguèt & Howe, 1973; Bächli et al. 1977; Bächli, Eichenberger & Hauri, 1978) and other cell types (Maeda et al. 1977) there is a rapid diffusion of viral antigens over the cell surface; the contrary, however, appears to be the case following fusion of '3-day' virus under conditions where cell swelling is prevented (Maeda et al. 1977). The freeze-fracture observations presented here show similar differences in the fate of incorporated viral envelope components. In contrast to the rapid diffusion (and eventual disappearance) of the smooth linear segments of viral envelope following fusion of '3-day' virions (Knutton, 1977), with '1-day' virus, not only are the smooth membrane elements stable for long periods of time but they actually aggregate together. Although the molecular composition of these linear elements of viral envelope is unknown it has been suggested that they are lipid bilayer membrane generated and stabilized by a linear association of 'reorganized' F (and possibly HN) envelope glycoprotein complexes (Knutton, 1978). If this is correct then further aggregation to form the long lengths of smooth viral membrane illustrated in this paper, once the constraint limited by the small size of individual virus particles is removed, would seem plausible. The suggestion of Volsky & Loyter (1978) that they are viral M protein (Hewitt & Nermut, 1977) cannot be correct because it is quite clear both from thin-section and freeze-fracture images that the linear components are invaginations of the entire viral envelope including the lipid bilayer.

Aggregation of elements of smooth linear viral membrane following fusion of '1-day' virions indicates that some lateral movement of incorporated viral envelope components must occur. However, this is clearly a different phenomenon to that observed following fusion of viral envelopes under conditions where cell swelling occurs. In this latter case a rapid diffusion of viral envelope components over the entire cell surface takes place. These differences in the fate of viral envelope components following virus envelope-cell fusion suggest that cell swelling is the factor responsible and that cell swelling perturbs the structure of the erythrocyte membrane, in some as yet undefined way, so as to allow the rapid lateral diffusion of incorporated viral membrane components.

Lateral movement of erythrocyte integral membrane proteins is thought not to occur or be extremely limited in intact erythrocytes (Singer, 1974) and morphological studies have led to the conclusion that integral membrane proteins are restricted by the spectrin-actin meshwork (Elgaaeter, Shotton & Branton, 1976; Shotton, Thomson, Wofsy & Branton, 1978). Disruption of the spectrin-actin meshwork during cell swelling (and haemolysis) could well be the perturbation of membrane structure which allows the unrestricted diffusion of, albeit incorporated, membrane proteins. Whether this applies to erythrocyte membrane proteins in general or to other cell types has yet to be determined.
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Many of the detailed observations and quantitative estimates of the lateral mobility of membrane proteins have been carried out using erythrocytes (Fowler & Branton, 1977) or other cell types (Frye & Edidin, 1970; Edidin & Wei, 1977) fused with Sendai virus under conditions where swelling occurred. Although these authors (Edidin & Wei, 1977) suggest that the effects of Sendai virus do not affect the observed diffusion rates, the results presented here clearly indicate that cell swelling, induced by Sendai virus, does affect diffusion rates at least for incorporated virus proteins. Consequently, this is a question which needs to be reconsidered. The use of ‘i-day’ Sendai virus will now allow observation and measurement of diffusion rates of membrane proteins to be obtained under conditions where cell swelling does not occur or is minimal.

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REFERENCES


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